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Controlled release of sphingosine-1-phosphate agonist with gelatin hydrogels for macrophage recruitment

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Abstract

The objective of this study is to design a drug delivery system (DDS) for the in vivo promotion of macrophage recruitment. As the drug, a water-insoluble agonist of sphingosine-1-phosphate type 1 receptor (SEW2871) was selected. The SEW2871 (SEW) was water-solubilized by the micelle formation with gelatin grafted by L-lactic acid oligomer. The SEW-micelles were mixed with gelatin, followed by the dehydrothermal crosslinking of gelatin to obtain gelatin hydrogels incorporating SEW-micelles. SEW was released from the hydrogels incorporating SEW-micelles in vitro and in vivo. The SEW water-solubilized showed an activity of in vitro macrophage migration. When implanted into the back subcutis or the skin wound defect of mice, the hydrogel incorporating SEW-micelles promoted the macrophage migration toward the tissue around the implanted site to a significantly great extent compared with SEW-free hydrogel and that mixed with SEW-micelles. The hydrogel is a promising DDS to enhance macrophage recruitment in vivo.

1. Introduction

Macrophage plays a critical role in inflammation process. If macrophage is depleted, the wound repair is often delayed [1, 2]. Macrophage deteriorates severe inflammation, while they properly suppress inflammation to induce tissue regeneration [3, 4]. It is, thus, recognized that the inflammatory condition is greatly influenced by the existence and phenotype of macrophage. The findings suggest that the existence and phenotype of macrophage modify the extent of inflammation which can regulate the process of tissue regeneration. It is conceivable that the enhanced recruitment of macrophage physiologically induces the inflammation, which starts the regeneration and repairing process for injured or damaged tissues although the quality of inflammation greatly affects that of healing process. However, the healing process will not start up without the suitable induction of inflammation. As the first trial to induce the inflammation, it will be essential to design a technology for enhancement of in vivo macrophage recruitment. The objective of this study is to develop the technology of macrophage recruitment and evaluate the extent of recruitment enhancement.

A “find me” signal is known to function as the chemoattractant for macrophage [5]. Apoptotic cells secrete the “find me” signal by which macrophage is recruited to the cells for their phagocytic exclusion. Sphingosin-1-phosphate (S1P) is one of “find me” signals [6]. However, mouse macrophage have three types of S1P receptors, S1PR1, S1PR2, and S1PR3[7-9]. When S1P binds to the S1PR1, macrophage migration is enhanced [8, 10]. Oppositely, when S1P binds to the S1PR2,

macrophage migration is suppressed. The SEW2871 (5-[4-Phenyl-5-(trifluoromethyl)thiophen-2-yl]-3-[3-(trifluoromethyl) phenyl] 1,2,4-oxadiazole, SEW) is a S1PR1-specific agonist that does not act on the S1PR2 [11]. The SEW is a good drug to induce the recruitment of macrophage [12, 13]. Moreover, it has the weaker potential of S1PR1 down-regulation than another agonist of FTY720-P [14]. However, considering the in vivo efficient usage of water-insoluble and low-molecular weight SEW, it is necessary to develop the pharmaceutical form of administration.

Gelatin is a biodegradable material and has been extensively used for food, pharmaceutical, and medical purposes. The biosafety has been proven through their long practical applications. Gelatin is a denatured form of collagen which is the most abundant component of extracellular matrix in the body tissue. The material itself and the product degraded are both biocompatible. The material advantages of gelatin are the easiness of chemical modification and the commercial availability of materials with different physicochemical properties. We have prepared hydrogels with different biodegradabilities from gelatin or the derivatives and succeeded in augmenting the therapeutic activities of water-soluble drugs, such as growth factors [15, 16], chemokine [17], plasmid DNA [18], anti-tumor drug [19], and siRNA [20]. It is found, in addition, that the hydrogel can release a water-insoluble drug in the controlled fashion [21].

In this study, the biodegradable gelatin hydrogel was designed for the controlled release of water-insoluble SEW. A hydrophobic derivative of gelatin was prepared by grafting L-lactic acid

oligomer (LAo) to gelatin. SEW was water-solubilized by the micelles formation with the gelatin grafted by LAo (LAo-g-gelatin) (SEW-micelles). The SEW-micelles were mixed with gelatin, followed by the dehydrothermal crosslinking in different conditions to obtain gelatin hydrogels incorporating SEW-micelles. We examine the in vitro and in vivo release of SEW from the hydrogels incorporating SEW-micelles. When the hydrogel incorporating SEW-micelles were implanted into the back subcutis of mice, the macrophage recruitment into the implanted site was evaluated comparing with that of free SEW-micelles.

2. Materials and Methods

2.1. Materials

Gelatin with an isoelectric point (pI) of 5.0 ($M_w = 100,000$), prepared via an alkaline process of bovine bone (pI5 gelatin) or with a pI of 9.0 ($M_w = 100,000$), prepared via an acid process of pig skin (pI9 gelatin) and collagenase L were kindly supplied from Nitta Gelatin Co., Osaka, Japan. Disuccinimidyl carbonate (DSC), 4-dimethylaminopyridine (DMAP), and other chemicals were purchased from Nacalai Tesque Inc., Kyoto, Japan. SEW was purchased from Cayman Chemical Co., Michigan.

2.2. Synthesis of L-Lactic acid oligomers

L-lactic acid oligomers (LAo) with different molecular weights were synthesized from L-lactide monomers by ring opening polymerization with a stannous octate catalyst and 1-dodecanol as an initiator [22]. Briefly, L-lactide (20 g, 138.8 mmole, Purac Biochemical BV, Gorinchem, Netherlands) was melted at 130 °C in a nitrogen atmosphere, followed by the addition of toluene (5.6 ml) containing 1-dodecanol (4.67 g, 24.6 mmole, 2.10 g, 11.0 mmole or 1.35 g, 7.1 mmole) and stannous octate (0.56 g, 1.38 mmole). After mixing for 4 hr at 130 °C, the reaction product was dissolved in chloroform and the solution was poured into ethanol for precipitation three times. After precipitation, the solution was freeze-dried. The number-averaged molecular weight of LAo

prepared was determined by ^1H NMR spectroscopy (JNM-EX, JEOL, Ltd., Tokyo, Japan).

2.3. Synthesis of gelatin grafted with LAo

The pI5 gelatin was dissolved in anhydrous dimethyl sulfoxide (DMSO, 30 ml) at room temperature. Various amounts of LAo with number-averaged molecular weights of 950, 2,200, and 3,200 (1.0 , 3.0 , and 5.0×10^{-5} mole) were dissolved in 10 ml DMSO, and then DSC and DMAP (1.0 , 3.0 , and 5.0×10^{-5} mole) were dissolved in 2.5 ml of DMSO. The solution was mixed for 3 hr under stirring at room temperature to activate the hydroxyl groups of LAo. The solution of activated LAo was slowly added to the gelatin solution, while the mixture was stirred overnight at room temperature for LAo grafting to gelatin. The product solution was dialyzed against double distilled water (DDW) with a dialysis tube with the cut-off molecular weight of 12,000–14,000 (UC 30-32-100, EIDIA Co., Ltd, Tokyo, Japan) for 72 hr at room temperature, followed by freeze-drying to obtain the LAo-grafted gelatin.

2.4. Measurement of critical micellar concentration of LAo-grafted gelatin

The critical micellar concentration (CMC) of LAo-grafted gelatin was determined by the conventional fluorescence technique with pyrene [23]. LAo-grafted gelatin was dissolved in 10 mM of phosphate-buffered solution (PBS, pH 7.4) at the concentrations from 0.001 to 1 mg/ml. Pyrene

was dissolved in acetone at 6 mM. After mixing with the pyrene solution, change in the emission intensity ratio (I₃₃₉/I₃₃₃) of mixed pyrene and polymer solution was measured at excitation wavelengths of 333 and 339 nm and the emission wavelength of 390 nm. The CMC was determined as the concentration where the intensity ratio was sharply changed on the ratio-polymer concentration plot. Experiments were done independently 3 times for each sample unless otherwise mentioned.

2.5. Dynamic light scattering measurement of LAO-grafted gelatin micelles and SEW-micelles

To measure the apparent molecular size of LAO-grafted gelatin micelles, dynamic light scattering (DLS) measurement was carried out on a DLS-DPA-60HD (Otsuka Electronic Co., Ltd, Osaka, Japan) equipped with a He–Ne laser at a detection angle of 90° at room temperature. Each sample was dissolved in DDW (1.0 mg/ml).

2.6. Water-solubilization of SEW by LAO-grafted gelatin

LAO-grafted gelatin solution (1.0 mg/ml) in DDW and SEW solution (1.0 mg/ml) in ethanol were prepared. The SEW solution (474 µl) was added to the LAO-grafted gelatin solution (9.0 ml), followed by 24 hr stirring at room temperature. The reaction mixture was centrifuged (8,000 rpm, 5 min, 4 °C) to separate water-insoluble SEW, and freeze-dried to obtain the SEW water-solubilized

by LAO-grafted gelatin micelles (SEW-micelles). To measure the amount of SEW incorporated into the SEW-micelles, acetonitrile was added to the SEW-micelle freeze-dried, followed by ultrasonication for 10 sec on ice to allow SEW to extract in acetonitrile. After centrifugation (10,000 rpm, 5 min, 4 °C), the amount of SEW in the supernatant was detected by high-performance liquid chromatography (HPLC, LC-20AT (Shimadzu Corp., Kyoto, Japan) and TSKgel ODS-100V 5 μ m (15 cm \times 4.6 mm) column (TOSOH Corp., Tokyo, JAPAN). The mobile solution was a mixed of water-acetonitrile (10:90, v/v) solution and the flow rate was 1.0 ml/min, while the absorbance of SEW was measured at the wavelength of 242 nm.

2.7. Preparation of gelatin hydrogels incorporating SEW-micelles

Hydrogels were prepared through the dehydrothermal crosslinking of gelatin together with SEW-micelles at 160 °C for 24 hr [24]. Briefly, pI9 gelatin in DDW (100 mg/ml, 1.0 ml) and SEW-micelles containing 7.5 or 75 μ g/ml of SEW in DDW (1.0 ml) were mixed. Then, the solution (2.0 ml) was cast into a polypropylene dish (20 mm \times 20 mm, Sakura Finetek Japan Co., Ltd, Tokyo, Japan), followed by freezing in liquid nitrogen and freeze-drying. The hydrogels freeze-dried were treated under vacuum, followed by heating at 160 °C for 24 hr.

2.8. In vitro test of SEW release from gelatin hydrogels incorporating SEW-micelles and hydrogel

degradation

The in vitro test was performed in PBS at 37 °C for the initial 24 hr, and thereafter in PBS containing collagenase until to 40 hr. The sampling times were 1, 2, 4, 8, 24, 25, 26, 28, 32, 36, and 40 hr. The gelatin hydrogel (5.0-5.5 mg) incorporating SEW-micelles containing about 3.8 µg/ml of SEW was placed in 500 µl PBS for the initial 24 hr, followed by the sampling to collect the supernatant at different time intervals, and fresh 500 µl PBS was added. The PBS (500 µl) was exchanged to 500 µl PBS containing 50 µg/ml collagenase, and then the similar release test was continued. The amount of SEW was measured by HPLC described above. For the in vitro degradation test, the PBS with or without collagenase was used by same way, and the sampling time was also same. The protein amount of supernatant sampled was determined by the micro bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) to evaluate the time course of hydrogel degradation.

2.9. In vivo test of SEW release from gelatin hydrogels incorporating SEW-micelles

C57BL/6CrSlc male mice (8-12 weeks old) were purchased from Shimizu Laboratory Supplies Co., Kyoto, Japan. All the animal experimentation was conducted in accordance with the guidance of the Institute for Frontier Medical Sciences, Kyoto University. For the in vivo release study, the hydrogel was implanted into the back subcutis of mice according to the method reported previously

[25]. Briefly, the back skin hair of mice was shaved by a razor under pentobarbital anesthesia (50 mg/kg). Then, the back skin was cut by a scissor and from the cut, the hydrogel (8 x 8x 2 mm³, 15-16 mg) incorporating SEW-micelles containing 12 µg of SEW was implanted into the back subcutis, and thereafter the back skin was sutured. At 1, 3, 7, 10, 14, and 21 days after hydrogel application, mice were sacrificed by the over-dose injection of anesthetic, and then the hydrogels were taken out to measure the SEW remaining by the HPLC measurement described above. The experiment was independently performed for 3 samples per experimental group at each sampling point.

2.10. In vitro migration assay

To evaluate the bioactivity of SEW for macrophage migration, mouse bone marrow-derived macrophage was used [26]. Briefly, the mouse bone marrow was collected from the femurs and tibia of mice by the conventional syringe aspiration method. The cells collected were resuspended in 10 ml of Iscove's Modified Dulbecco's Medium (IMDM) containing 20 vol% fetal calf serum (FCS) and 50 ng/ml recombinant human macrophage-colony stimulating factor (M-CSF), and cultured on 10-cm diameter dishes in 37°C, 5% CO₂-95% air atmosphere for 6 days. The cells cultured were used as mouse bone marrow derived macrophage. The migration of macrophage was evaluated by the conventional method with a modified Boyden chamber equipped with a polycarbonate filter (5

μm pores; Corning, NY, USA) [6]. Briefly, cells (5×10^4 cells/well) were added to the upper compartment, while the lower chamber was filled with FCS-free culture medium containing different amounts of free SEW and different SEW amount of SEW-micelles released from the hydrogels. For free SEW, SEW solution (1.0 mg/ml) in ethanol was added into the medium to give different amounts. Cells were allowed to migrate for 3 hr, at 37 °C, and then the non-migrating cells present on the top of filter were removed mechanically. The migrated cells on the bottom of filter were fixed, and stained with Hemacolor ® (Merck Millipore, Darmstadt, Germany), to count the number of cells migrated for 12 areas of filter, randomly selected.

2.11. Evaluation of macrophage recruitment by hydrogels incorporating SEW-micelles in the subcutis of mice

Mice were anaesthetized by the intraperitoneal administration of pentobarbital (50 mg/kg). The hydrogels incorporating SEW–micelles containing 1 (SEW1Gel) and 10 μg (SEW10Gel) of SEW were implanted into the back subcutis of mice. The control samples used were SEW-free gelatin hydrogels, ethanol solution of 10 μg SEW, SEW-micelles containing 10 μg of SEW, (SEW10 μg -micelles solution), and PBS alone. At different time intervals, the tissue around the hydrogel implanted and the hydrogel were collected and digested in 2 mg/ml of collagenase D at 37°C for 1.5 hr, followed by the collection of cell fractions through at 40 μm -aperture strainers (BD Biosciences,

San Jose, California). The tissues and hydrogels were separated manually by before collection through the 40 μ m-aperture strainers. The cell suspensions obtained were lysed in 500 μ l of lysis buffer (555899, BD Biosciences, San Jose, California) for 15 min at 37°C. After washing twice by 500 μ l of PBS, the cells were treated by fluorescent-labeled FITC 7-AAD (BD Biosciences, San Jose, California), CD45 (eBioscience, Inc., San Diego, California), Ly6G (eBioscience, Inc., San Diego, California), and CD11b (BD Biosciences, San Jose, California), F4/80 antibodies (Biolegend, Inc., San Diego, California). The cells labeled were analyzed by flow cytometry (FACSCanto II flow cytometer, BD Biosciences, San Jose, California). The cells with CD45⁺ Ly6G⁻ CD11b⁺, and F4/80⁺ were defined as macrophage. The number of animals used for each experimental time point were 3.

2.12. Evaluation of macrophage recruitment with a mouse model of skin wound

To evaluate macrophage recruitment to the inflammatory site, a mouse model of skin wound was prepared [27]. Mice were anaesthetized by the intraperitoneal administration of pentobarbital (50 mg/kg). The back hair of mice was shaved. The skin defect was made on the back subcutis by 8 mm-size biopsy punch. The gelatin hydrogel (8 x 8x 2 mm³, 15-16 mg) alone (Gel) and SEW10Gel were applied and sutured to the surrounding skin. SEW 10mg-micelles solution was applied to the defect, while the defect was not covered by Gel. At different time intervals, the tissue around wound

area and hydrogel implanted were collected and digested in 2 mg/ml of collagenase D at 37°C for 1.5 hr, followed by the collection of cell fractions through at 40 µm strainers (BD Biosciences, San Jose, California). The cells were treated with FITC-labeled antibodies and analyzed by the flow cytometry procedure described above.

2.13 Statistical analysis

All the results were expressed as the mean \pm standard deviation (SD). Significant analysis was done based on the one-way ANOVA, and the difference was considered to be significant at $P < 0.05$.

3. Results

3.1. Characterization of LAo-grafted gelatin

Table 1 summarizes the physicochemical properties of LAo-grafted gelatin prepared in different conditions. Every LAo-grafted gelatin showed a CMC value, which indicates property of micelles formation. The apparent molecular size LAo-grafted gelatin micelles ranged from 142 to 379 nm. The water-solubility of LAo-grafted gelatin tended to become smaller with an increase in the molecular weight of LAo grafted and the grafted ratio (data not shown).

3.2. Water-solubilization of SEW by LAo-grafted gelatin

Figure 1 shows the percentage of SEW water-solubilized by LAo-grafted gelatin. The amount of SEW water-solubilized depended on the molecular weight of LAo grafted and the grafted ratio. Based on the results, the LAo2-1 of LAo-grafted gelatin was used for the following experiments.

3.3. SEW release from gelatin hydrogels incorporating SEW–micelles and hydrogel degradation

Figure 2 shows the time profiles of hydrogel degradation and SEW release from gelatin hydrogels incorporating SEW–micelles in PBS with or without collagenase. In the presence of collagenase, SEW was released with time from the gelatin hydrogels. On the contrary, only 4 % of SEW release was observed in collagenase-free PBS (Figure 2A). In PBS containing collagenase, the hydrogels

were degraded with time, whereas little hydrogel degradation was observed in PBS (Figure 2B). A good correlation in the time profiles was observed between the SEW release and hydrogel degradation. Figure 3 shows the SEW was released gradually from the hydrogels incorporating SEW-micelles in vivo.

3.4. Biological activity of SEW and SEW-micelles released from gelatin hydrogels in vitro

Figures 4(A) and (B) shows the macrophage migration by different amounts of free SEW and different amounts of SEW released from hydrogels incorporating SEW-micelles for 2 and 16 hr incubation in PBS containing collagenase. The percentage of migrated cells in the FCS-free culture medium without SEW was 2.0 %. On the contrary, significantly enhanced macrophage migration was observed at 0.1 or higher concentrations of SEW. The SEW released from the hydrogels also enhanced the macrophage migration, irrespective of the SEW amount incorporated and the time period released.

3.5 Biological activity of SEW and gelatin hydrogels incorporating SEW-micelles

Figure 5(A) shows the macrophage recruitment 12 and 24 hr after implantation of gelatin hydrogels incorporating SEW-micelles. The total number of cells migrated into the sponges was about 1.4 and 2.1×10^4 cells 12 and 24 hr after treatment, respectively. Significant enhancement of

macrophage recruitment was detected for both the SEW1Gel and SEW10Gel. On the contrary, no recruitment enhancement was observed for the SEW-free gelatin hydrogels.

Figure 5(B) shows the macrophage recruitment 12 and 24 hr after implantation of gelatin hydrogels incorporating SEW-micelle. The total number of cells and the tissue collected was 5.4 and 5.8×10^3 cells for 12 and 24 hr after treatment, respectively. An enhanced recruitment was observed for the hydrogels incorporating SEW-micelles. Macrophage recruitment was not enhanced for the Gel, SEW10 μg solution, and SEW10 μg -micelles solution.

Figures 6 and 7 show the time courses of the number ratio of macrophage/total cells infiltrated into the tissue around hydrogels at the normal tissues and the skin wound. For the normal tissue, significant enhancement of macrophage recruitment was observed at the tissue around the SEW10Gel implanted 12 hr later. On the other hand, when the SEW10Gel were implanted into the skin wound, macrophage was recruited into the tissue around the hydrogels 1 day after implantation to a significantly great extent compared with Gel+10mg SEW-micelles solution and gelatin hydrogels alone.

4. Discussion

The present study demonstrates that the biodegradable hydrogel can release the water-insoluble SEW of biological activity. SEW was water-solubilized by the micelles formation with LAo-grafted

gelatin with a CMC value. The percent water-solubilized was changed by altering the molecular weight of LAo grafted and the grafted ratio. It is likely that the difference in the molecular weight of LAo and the grafted ratio causes the different interaction of LAo components and SEW, resulting in varied SEW incorporation into the micelles. The micelles stabilization and the amount of SEW solubilized in water are influenced by the hydrophilic-hydrophobic balance of LAo-gelatin and the interaction between the SEW and LAo-hydrophobic core. LAo2-1 may form the micelle with the inner environment of an appropriate hydrophobicity which can entrap SEW. Since gelatin hydrogels are not degraded in collagenase-free PBS, SEW is not released from the hydrogels (Figure 2). Gelatin is normally degraded by enzymes, but not by a simple hydrolysis. In the presence of collagenase, the hydrogels would be degraded enzymatically to generate water-soluble gelatin fragments, resulting in SEW release due to the water solubilization of SEW associated with the gelatin fragment. A good correlation in the time profile between the SEW release and the hydrogel degradation was observed. In the hydrogel system, the SEW is released from the hydrogel only if the hydrogel is degraded to generate water-soluble gelatin fragments. However, a certain amount of SEW is initially released because SEW-micelles immobilized with non-crosslinked, free gelatin molecules are released out by the simple diffusion. Most of SEW-micelles immobilized with gelatin molecules crosslinked are released out of hydrogel as a result of gelatin water-solubilization due to the hydrogel degradation. This is the mechanism of SEW-micelles release from the hydrogel. In the

collagenase-free PBS, the gelatin hydrogel is not enzymatically degraded. In the release test in collagenase-containing PBS the gelatin hydrogel is degraded with time while the SEW-micelles are released accompanied with the degradation. The rates of SEW release evaluated from the in vitro release study were in good accordance with those of hydrogel degradation. This finding indicates that the SEW release is governed only by the degradation of hydrogels as the release carrier. The hydrogels achieved the controlled release of SEW even in vivo (Figure 3). It is likely that the SEW release in vivo was achieved through the enzymatic degradation of gelatin hydrogels similarly to the in vitro condition of collagenase-containing PBS.

SEW and the SEW released from the hydrogels enhanced the in vitro macrophage migration (Figures 4(A) and (B)). This indicates that the activity of SEW remains even after the dehydrothermal treatment for hydrogel preparation, and the SEW released in the SEW-micelles form was also bioactive. The HPLC study revealed that the SEW released showed a peak similar to that of original SEW in terms of the peak position and shape (data not shown).

The hydrogels incorporating SEW-micelles showed significant enhancement of macrophage recruitment in vivo, whereas the SEW-free hydrogels and SEW-micelle solution did not (Figures 5(A) and (B)). Considering the in vivo profile of SEW release, it is conceivable that the concentration gradient of SEW in the tissue formed by the release plays an important role in the enhancement of macrophage recruitment. The significant enhancement of macrophage recruitment

was observed for both the normal and wound skins, although the time profiles of macrophage recruitment was different (Figures 6 and 7). The macrophage recruitment would be influenced by the tissue inflammation other than the SEW released. This is because macrophage is generally recruited and play a critical role in the early stage of inflammation [2].

To evaluate the effect of SEW release profile on the in vivo macrophage recruitment, different types of gelatin hydrogels with different time periods of SEW release for 3, 9 or 21 days, were prepared, and the effect on the macrophage recruitment was assessed (Figure S2). As the result, the enhancement of macrophage recruitment was observed only for the hydrogels incorporating SEW-micelles for 21 days release. This may be explained in terms of difference in the SEW release profiles. An initial burst in SEW release from hydrogels releasing SEW for 3 and 9 days was large compared with those releasing SEW for 21 days. If the initial burst is too large and the drug is localized around the hydrogel only for a short time period, the concentration of SEW near the hydrogel will become rapidly low. It is possible that the rapid lowering SEW concentration results in reduced microphages recruitment. Histological evaluation was performed by the H&E and immunohistochemical staining methods. Unfortunately, however, the difference in the macrophage recruitment among the groups was not always demonstrated by the histological evaluation (data not shown). As the crosslinking extent of the hydrogels increases, the time period of SEW release tended to be longer in the back subcutis of mice (Figure S1). It is highly conceivable that the longer in vivo

release of SEW generates the large gradient of SEW concentration around the hydrogel, resulting in the enhanced macrophage recruitment in vivo.

In conclusion, SEW was water-solubilized by the micelles of LAO-grafted gelatin. The SEW water-solubilized was released from the gelatin hydrogels. In vivo macrophage recruitment was enhanced by the controlled release of SEW from the gelatin hydrogels. The SEW concentration and the concentration gradient to increase the macrophage recruitment suitable for the therapeutic effects are not clear at present. Further study is needed to evaluate the effect of macrophage recruitment and their phenotype on the extent of tissue regeneration.

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Figure 1. Percentage of SEW water-solubilized by various LAO-gelatin samples.

Figure 2. The time profiles of gelatin hydrogels incorporating SEW micelles degradation (A) and SEW release from the hydrogels incorporating SEW micelles (B). The release test was performed at 37 °C in PBS for the initial 24 hr, and thereafter in PBS containing collagenase.

Figure 3. The time profile of in vivo SEW release from gelatin hydrogels incorporating SEW micelles in the back subcutis of mice.

Figure 4. Macrophage migration of SEW and that released from hydrogels incorporating SEW-micelles. (A) The effect of SEW concentration on the number of macrophage migrated. (B) SEW released from hydrogels incorporating SEW-micelles for 2 and 16 hr in PBS containing collagenase. The concentration of SEW released was adjusted at 0.1 and 1 μ M based on the HPLC measurement. The number of macrophage migration is defined as 1 to calculate the migration ratio for samples. * $p < 0.05$; significant against the value at the SEW concentration of 0.

Figure 5. (A) The number ratio of macrophage/total cells recruited into the hydrogels incorporating 1 and 10 μ g of SEW or SEW-free hydrogels 12 hr (\square) and 24 hr after implantation (\blacksquare); SEW-free

hydrogels (Gel), hydrogels incorporating SEW-micelles containing about 1.0 $\mu\text{g/ml}$ of SEW (SEW1micelle) or SEW-micelles containing about 10 $\mu\text{g/ml}$ of SEW (SEW10micelle). (B) The number ratio of macrophages/total cells recruited at the tissue treated by gelatin hydrogels incorporating SEW-micelle, SEW-micelle or SEW solution 12 (\square) and 24 hr after implantation (\blacksquare): SEW-free hydrogels (Gel), hydrogels incorporating 1 μg (SEW1Gel) or 10 μg of SEW (SEW10Gel), 10 μg of SEW-micelle (SEW10micelle), and 10 μg of SEW solution (SEW solun). The number of macrophages migrated at the tissue 12 hr after Gel implantation is defined as 1 to calculate the migration ratio for samples. The number of animals used for each experimental time point were 3. * $p < 0.05$; significant against the value of Gel group at the corresponding implantation period.

Figure 6. Time courses of the percent of macrophage/total cells infiltrated at the tissue around hydrogels incorporating 1 and 10 μg of SEW or SEW-free hydrogels after implantation of Gel (\circ), SEW1Gel (Δ), SEW10Gel (\square), and Gel+10mg SEW-micelles (\bullet) on the subcutis of mice. * $p < 0.05$; significant against the value of Gel group at the corresponding time.

Figure 7. Time courses of the percent of macrophage/total cells ratio infiltrated into the inflammatory tissue after implantation of Gel (\circ), SEW1Gel (Δ), SEW10Gel (\square), and Gel+10mg SEW-micelles (\bullet) into the skin wound. * $p < 0.05$; significant against the value of Gel group at the corresponding time.

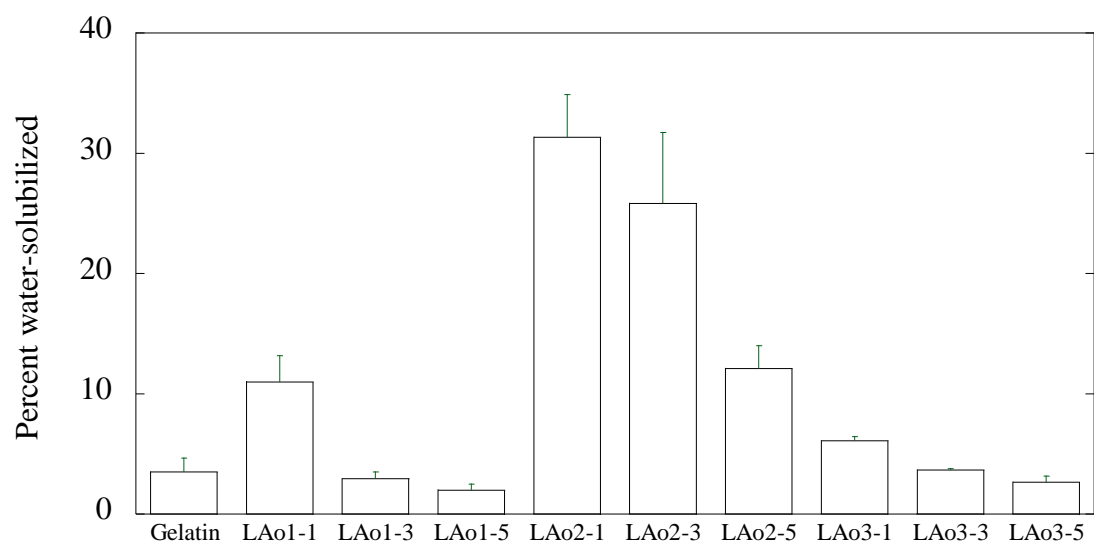


Figure 1.

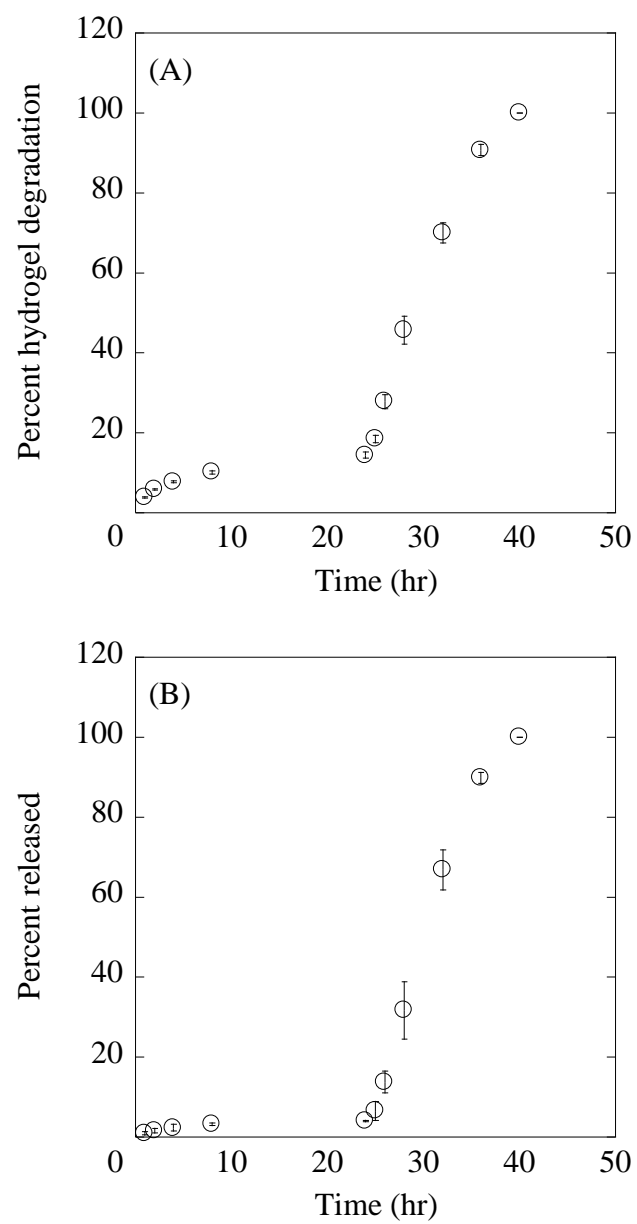


Figure 2.

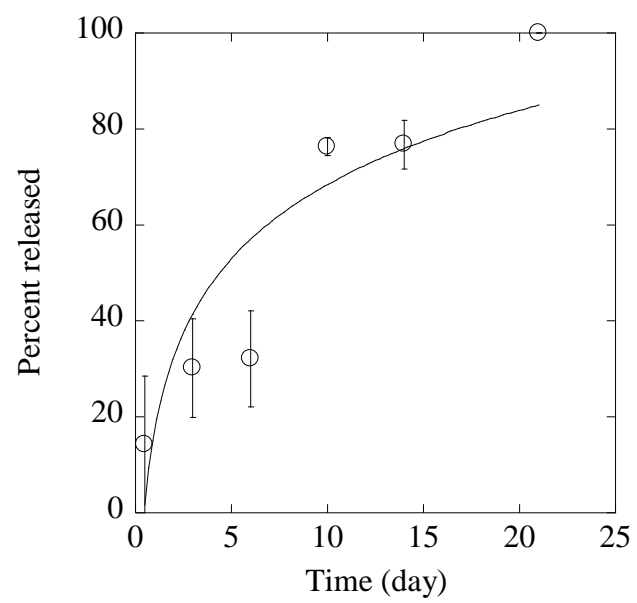


Figure 3.

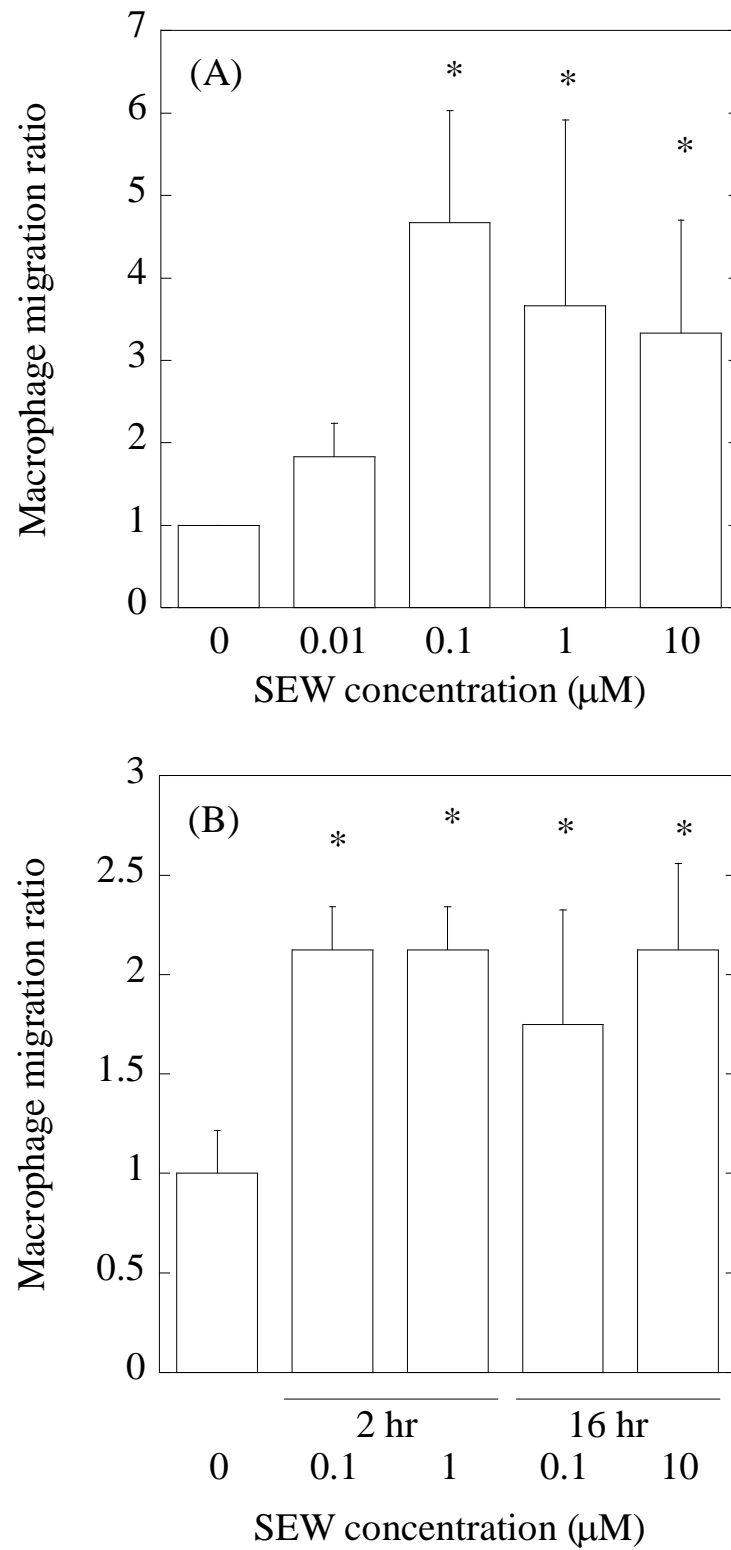


Figure 4.

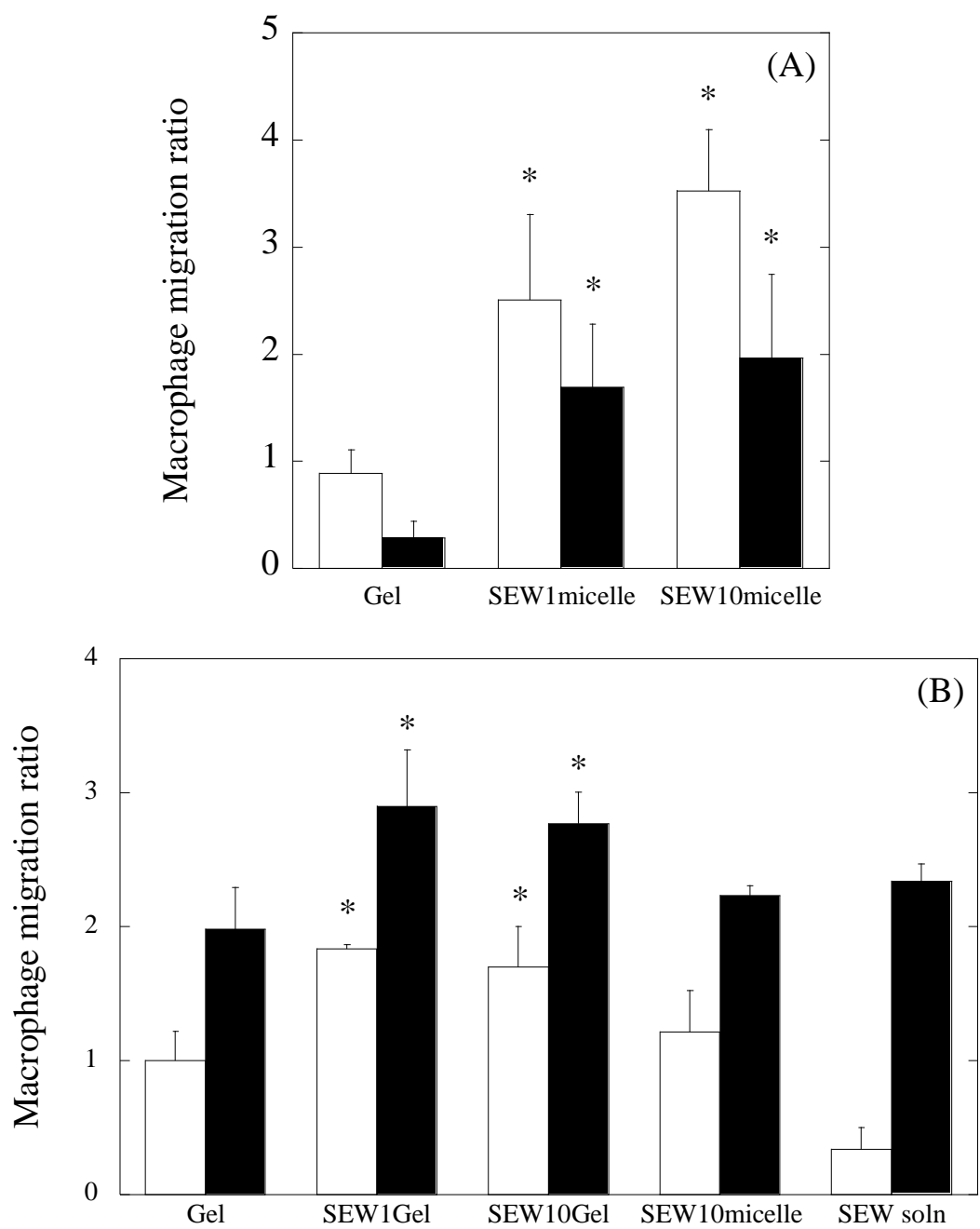


Figure 5.

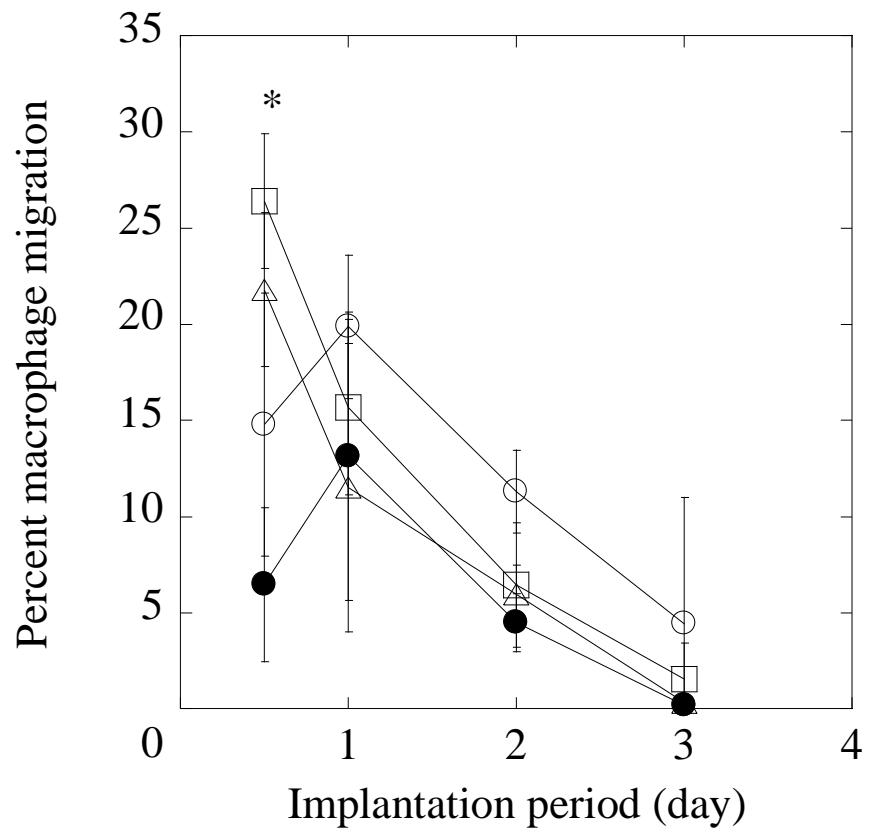


Figure 6.

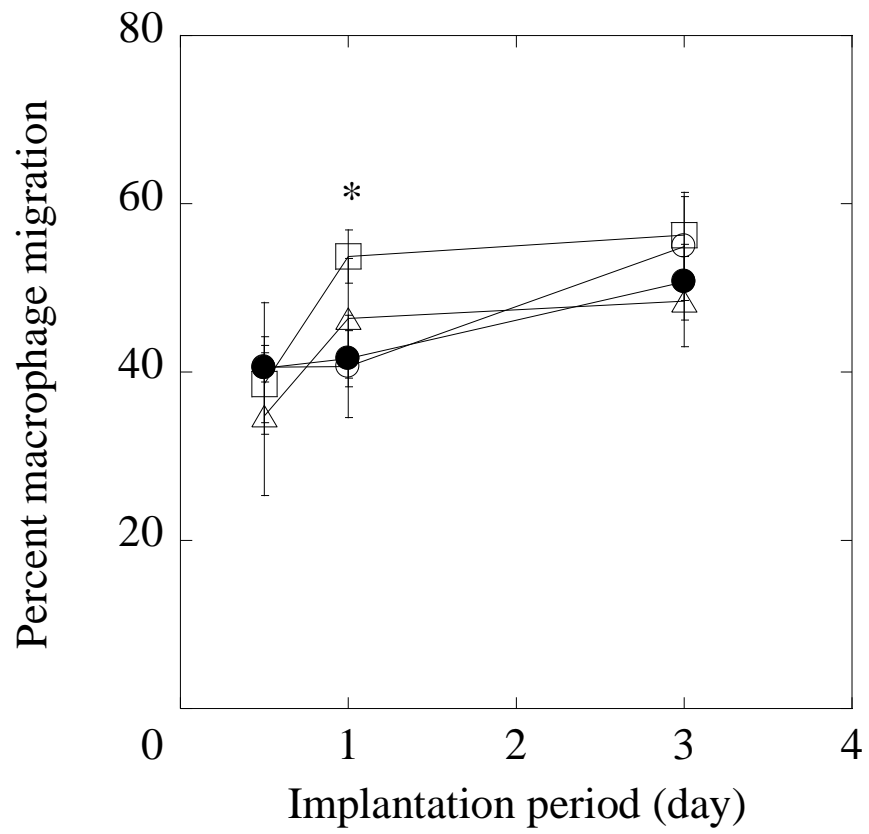


Figure 7.

Table1. Preparation and characterization of Lao-grafted gelatin.

Code	LAo used for grafting		CMC ($\mu\text{g/ml}$)	Apparent molecular size (nm)
	Mn	The ratio of LAo grafted (mole/mole gelatin)		
LAo1-1	950	1	39.9	$142.4 \pm 40.6^{\text{a}}$
LAo1-3	950	3	18.0	189.2 ± 41.4
LAo1-5	950	5	99.2	360.2 ± 83.8
LAo2-1	2200	1	21.4	245.7 ± 127.8
LAo2-3	2200	3	61.3	319.9 ± 157.6
LAo2-5	2200	5	31.6	215.2 ± 128.9
LAo3-1	3200	1	85.8	215.5 ± 43.4
LAo3-3	3200	3	40.8	230.6 ± 44.8
LAo3-5	3200	5	32.7	378.9 ± 85.5

^{a)} mean \pm SD

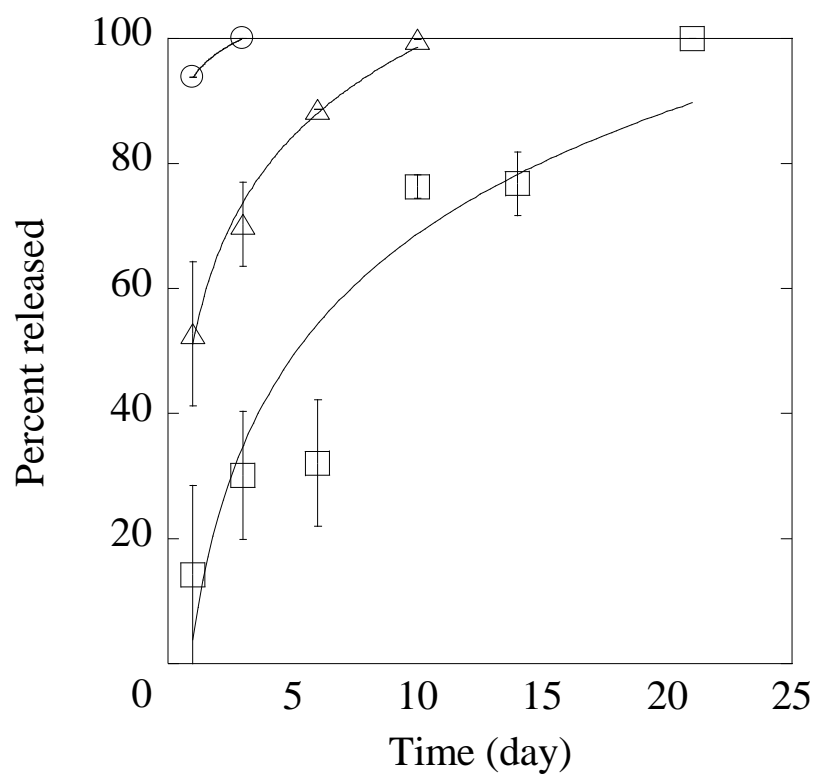


Figure S1. The time profile of in vivo SEW release from gelatin hydrogels incorporating SEW micelles in the back subcutis of mice: The hydrogels were prepared at 160 °C for 6 (○), 12 (Δ), and 48 hr (□).

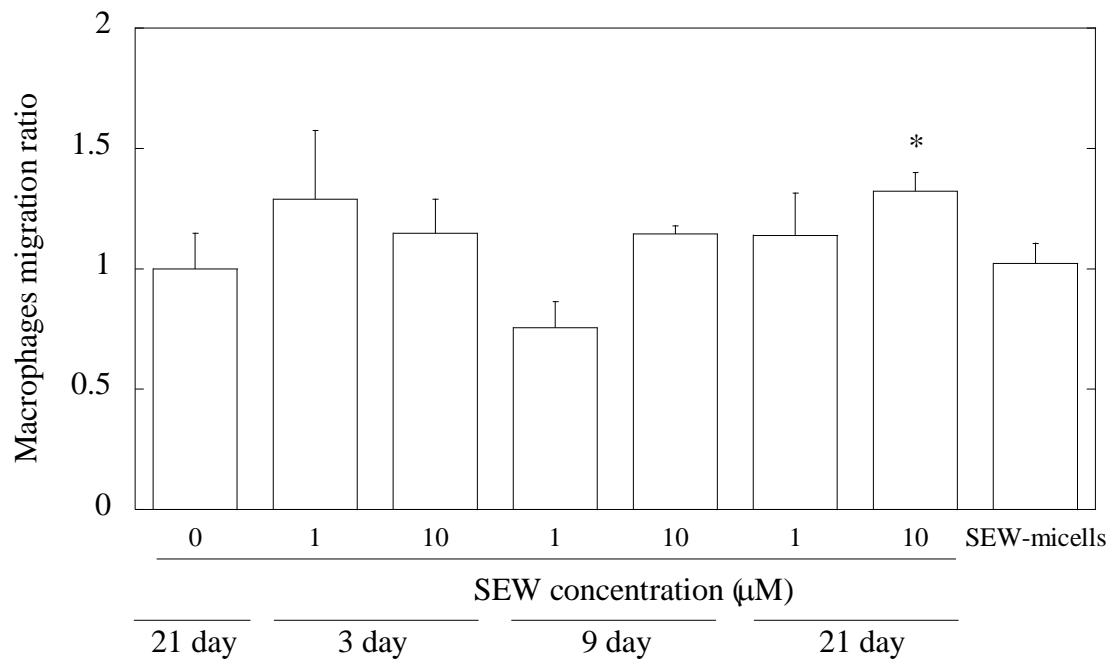


Figure S2. The number ratio of macrophage to total cells of SEW and that released from hydrogels incorporating SEW-micelles 24 hr after treatment: SEW-free hydrogels (0 - 21 day), hydrogels incorporating 1 μg, and 10 μg of SEW at different hydrogels' degradation period, and 10 μg of SEW-micelles. * $p < 0.05$; significant against the value at the SEW concentration of 0.